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Effect of NO2 Inhalation and Vitamin C Deficiency on Protein and Lipid Accumulation in the Lung

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16. ABSTRACT

Vitamin C-deficient and normal guinea pigs were exposed to various concentrations of NO_2 or air, and lavage fluid was obtained and analyzed for protein and lipid content. Exposure of normal animals to 752, 1880, 5640, or 9400 μg NO_2/m^3 (0.4, 1.0, 3.0, or 5.0 ppm) for 72 hr did not alter the protein or lipid content of lung lavage fluid. However, exposure of vitamin C-deficient animals to the same concentrations of NO_2 caused marked increases in lavage proteins and lipids at all but the 752 $\mu g/m^3$ (0.4 ppm) level. At 9400 μg NO_2/m^3 (5.0 ppm), 50% of the exposed vitamin C-deficient animals died, and pathologic study of the lungs showed proteinaceous edema fluid in the alveoli. Lungs from air-exposed animals and normal animals exposed to NO_2 appeared healthy. No effects were seen at 752 μg NO_2 (0.4 ppm) in either normal or deficient animals even when the time of exposure was extended to 1 week. At 9400 μg NO_2/m^3 (5 ppm) effects could be seen in vitamin C-deficient animals even when the exposure period was shortened to 3 hr. Assessment of protein and lipid content of lavage fluid provided a sensitive method for determining subtle changes in the lung following NO_2 exposure.

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Effect of NO_2 Inhalation and Vitamin C Deficiency on Protein and Lipid Accumulation in the Lung[1]

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Vitamin C-deficient and normal guinea pigs were exposed to various concentrations of NO_2 or air, and lavage fluid was obtained and analyzed for protein and lipid content. Exposure of normal animals to 752, 1880, 5640, or 9400 μg NO_2/m^3 (0.4, 1.0, 3.0, or 5.0 ppm) for 72 hr did not alter the protein or lipid content of lung lavage fluid. However, exposure of vitamin C-deficient animals to the same concentrations of NO_2 caused marked increases in lavage proteins and lipids at all but the 752 $\mu g/m^3$ (0.4 ppm) level. At 9400 μg NO_2/m^3 (5.0 ppm), 50% of the exposed vitamin C-deficient animals died, and pathologic study of the lungs showed proteinaceous edema fluid in the alveoli. Lungs from air-exposed animals and normal animals exposed to NO_2 appeared healthy. No effects were seen at 752 μg NO_2 (0.4 ppm) in either normal or deficient animals even when the time of exposure was extended to 1 week. At 9400 μg NO_2/m^3 (5 ppm) effects could be seen in vitamin C-deficient animals even when the exposure period was shortened to 3 hr. Assessment of protein and lipid content of lavage fluid provided a sensitive method for determining subtle changes in the lung following NO_2 exposure.

INTRODUCTION

Outdoor concentrations of NO_2 often reach levels of 1000 $\mu g/m^3$ (0.53 ppm) or greater for 1-hr periods in certain U.S. cities (EPA, 1977). The major source of this pollution is combustion of fossil fuel. Indoor concentrations up to 2000 $\mu g/m^3$ (1.06 ppm) have been reported in the vicinity of gas stoves (WHO, 1977), and cigarette smoking represents brief exposure to much higher levels of NO_2 (Bokhoven and Niessen, 1961). As pressure mounts to increase the use of fuels such as coal and diesel fuel, which often have a high yield of NO_2 per energy unit, the concentration of NO_2 encountered in such short term exposures, as well as the incidence, is likely to increase (Dawson and Schenker, 1979). The current U.S. ambient air quality standard for NO_2 of 100 $\mu g/m^3$ (0.05 ppm) annual arithmetic mean allows these short-term peak episodes to occur. There is a need to assess the health effects of such exposures so that a short-term standard can be developed.

Several studies have indicated that NO_2 causes edema and/or increased protein content in the air spaces, possibly due to damage of the respiratory epithelium (particularly the tight junctions) and capillaries or to some metabolic changes

[1] Disclaimer: This report has been reviewed by the Health Effects Research Laboratory, U.S. Environmental Protection Agency, and approved for publication. Mention of trade names or commercial products does not constitute endorsement or recommendation for use.

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(Sherwin and Carlson, 1973; Sherwin and Rickters, 1971; Sherwin and Layfield, 1976; Hine *et al.*, 1970; Nakajima *et al.*, 1972). Sherwin and Carlson (1973) demonstrated excess protein in lung lavage fluids using vitamin C-deficient guinea pigs exposed to 752 μg NO_2/m^3 (0.4 ppm) for 1 week. This was a particularly interesting observation since few health effects have been shown at this low level of NO_2.

These studies seemed worth pursuing since nearly half the population in the United States receives less than the recommended daily allowance of vitamin C (PHS, 1979). Also, there are a number of conditions in the human population which cause individuals to have blood levels of vitamin C which are 30–50% lower than normal. These include use of oral contraceptives (Wynn, 1975; McLeroy and Schendel, 1973), tetracycline treatment (Windsor *et al.*, 1972), and old age (Baker *et al.*, 1979). Smokers average 40% lower ascorbic acid plasma levels than nonsmokers, with heavy smokers having lower levels than moderate smokers (Brook and Grimshaw, 1968; Pelletier, 1968). This is particularly relevant since smoking produces very high levels of NO_2 (Bokhoven and Niessen, 1961). Individuals with these lowered levels of blood vitamin C do not appear to be ill but may be more susceptible to adverse conditions, including inhalation of photochemical oxidants.

Since vitamin C plays an important role in collagen production and vascular permeability (Barnes, 1975; Moran and Green, 1979) and since NO_2 has been reported to denature collagen and damage respiratory epithelium (Buell *et al.*, 1966; Buell, 1970; Hatton *et al.*, 1977), it is reasonable to postulate that a deficiency in vitamin C might enhance any NO_2-induced damage to the lung. The present study was conducted to test this hypothesis by determining the protein and lipid content of lung lavage fluids following exposures to various regimens of NO_2 and by comparing the effects in vitamin C-deficient and normal guinea pigs.

MATERIALS AND METHODS

Animals and diet. Male Hartley COBS guinea pigs weighing 300–350 g were obtained from Charles River Farms, Wilmington, Massachusetts, and used within 6 weeks of arrival. Animals were given food and water *ad libitum*. Normal animals were fed Wayne Guinea Pig Diet (Allied Mills, Inc., Chicago, Ill.), which is supplemented with 1000 ppm vitamin C. To make animals vitamin C deficient they were fed Wayne Rabbit ration (not vitamin C supplemented) for 2–5 weeks prior to and during exposure. This was preferable to feeding commercial preparations specially formulated to produce vitamin C-deficient guinea pigs because the animals accepted the rabbit chow more readily and the degree of deficiency obtained with rabbit chow was less severe and more representative of the level of deficiency commonly seen in the human population. Due to the variability in different lots of feed, efforts were made to standardize, as much as possible, by milling date of the feed used for these studies, and each group of animals was sampled for blood vitamin C levels to ensure that animals fed rabbit chow were indeed deficient.

Exposure to NO_2. Animals were exposed to NO_2 in individual stainless-steel wire mesh modules placed in Rochester-type chambers (Hinners *et al.*, 1968). Control animals were exposed to filtered room air in the same type of modules and

chambers at the same time that NO_2 exposures were taking place. NO_2 flow was initiated from the manifold of a cylinder containing 0.1% NO_2 in nitrogen, mixed with filtered room air at a point between the inlet damper and metering orifice and introduced into the chamber at a rate of one air change per minute. A mass flow controller was used to control the amount of NO_2 entering the chamber. The concentration of NO_2 was monitored continuously using chemiluminescent NO_2 analyzers (Bendix, Lewisburg, W. Va.), which were calibrated by the gas phase titration technique (EPA, 1975).

Experimental design. Three different continuous NO_2 exposure regimens were employed. In the first experiments, normal and vitamin C-deficient guinea pigs were exposed to 752, 1880, 5640, and 9400 μg NO_2/m^3 (0.4, 1.0, 3.0, and 5.0 ppm) for 72 hr. During this exposure period the maximum deviations from these mean NO_2 concentrations were $\pm 7\%$, $\pm 6\%$, $\pm 3\%$, and -12%, respectively. The time interval used was shorter and the range of concentrations wider than those employed by Sherwin and Carlson (1973). The second exposure regimen extended to 1 week the time of exposure of C-deficient and normal animals to the lowest previously tested concentration of NO_2 (752 $\mu g/m^3$—maximum deviation $\pm$ 6%). The third regimen shortened to 3 hr the time of exposure of C-deficient animals to the highest previously tested concentration of NO_2 (9400 $\mu g/m^3$—maximum deviation -12%). The second and third experimental regimens were carried out to see what effect varying the time interval would have on positive and negative effects seen in the first study. Following exposures, lavage fluid was obtained from all animals and analyzed for proteins and lipids.

Obtaining lavage fluid. Immediately after exposure or at designated times thereafter, guinea pigs were anesthetized with an intraperitoneal inoculation of nembutal, blood was obtained in heparinized vacutainers by cardiac puncture for ascorbic acid analysis, and the animals were exsanguinated by severing the abdominal aorta. The trachea was immediately exposed, a slit made just below the larynx, and a catheter inserted and secured with light-weight cord. Ten milliliters of warmed (37°C) saline (0.85%) was then injected into the lungs, allowed to remain for 1 min, withdrawn, and then injected and withdrawn four additional times. Repetitive lavage of the same solution was used because it resulted in higher concentration of protein in the lavage fluid. Cells were removed from lavage fluid by light centrifugation and discarded. A few red cells were usually visible in the cell pellet; however, the amount seen was relatively consistent regardless of treatment group. Fluids were stored at -20°C. Prior to lipid and protein analyses, lavage fluids were centrifuged at 27,000*g* (15,000 rpm) for 20 min to remove cellular debris.

Protein analyses. Protein concentrations were determined by the Lowry Method (Lowry *et al.*, 1951). Proteins in lung lavage fluids were also analyzed by polyacrylamide gel electrophoresis (PAGE) in 7% discontinuous cylindrical gels (Chrambach and Rodbard, 1971). Approximately 20 μg protein in 100 μl of lavage fluid containing 10% sucrose and a trace amount of bromophenol blue was added to individual tubes with a Hamilton syringe. The gels were electrophoresed for 2 hr at a current of 2.5 mA per gel. Gels were removed from the tubes and stained with Coomassie brilliant blue as previously described (Hu *et al.*, 1977) and de-

stained in a mixture of methanol, acetic acid, and water (10:3:27) until the background was cleared. Gels were photographed and scanned for relative quantitation of each peak. Scanning was carried out in a Gilford Model 250 spectrophotometer attached with a Model 2520 gel scanner.

Lipid analyses. For lipid analyses aliquots of 1.5 ml of lavage fluid were extracted using a modification (Hanson and Olley, 1963) of the Bligh and Dyer method (1959). To the lavage was added 5.64 ml of a mixture of methanol:chloroform (2:1). The resulting solution was vortexed for 30 sec and allowed to stand 10 min. Chloroform (1.89 ml) was added, followed by 1.89 ml of water with a 15-sec vortex after each addition. The biphasic mixture was then centrifuged for 10 min at 1500 rpm and the top layer discarded by aspiration. An aliquot (3.5 ml) of the bottom layer was evaporated under a stream of nitrogen at 40°C and the residue reconstituted with a known volume (300 μl) of chloroform:methanol (2:1).

Quantitation of the neutral lipids, cholesterol (Ch),[2] cholesterol esters (ChE), and free fatty acids (FFA) was accomplished using a modification of the procedure described by Touchstone *et al.* (1978). Cholesterol and cholesterol stearate were supplied by Sigma Chemical Company, St. Louis, Missouri, and oleic acid by the Applied Science Division, Milton Roy Company Laboratory Group, State College, Pennsylvania. Thin-layer chromatographic plates were 20 × 20 cm of 250 μm silica gel G thickness and were purchased from E. Merck, Darmstadt, W. Germany. They were scored into 10-mm-wide lanes with a Schoeffel scoring device (Schoeffel Instrument Division, Kratos, Inc., Westwood, N.J.), prewashed in the mobile phase of hexane: diethyl ether: acetic acid (80:20:1), and allowed to dry for at least 5 min before spotting. Plates were spotted according to the data-pairing technique (Bethke *et al.*, 1974) with 4 to 40 μl using an automatic 18-place spotter (Analytical Instrument Specialties, Inc., Libertyville, Ill.) at 60°C and under a stream of N_2. After development, components were visualized by spraying the plates with a solution of 30 g/liter of cupric acetate in phosphoric acid (80 ml/liter) and heating at 190° for 15 min. The density of the standard and sample spots were assayed by spectrodensitometry (Schoeffel Model 3000 spectrodensitometer and 300D density computer) at 365 nm in the single-beam reflectance mode using a 10-mm beam width. Standard curves of peak area versus the square root of the mass were generated by digitization (Model 9864A digitizer, Hewlett–Packard, Palo Alto, Calif.) and sample amounts determined from the standard regression curves.

Ascorbic acid analysis. The method described by Roe (1954), with slight modification, was used to determine the amount of ascorbic acid in the blood. Briefly, 0.3 ml blood was added to 1.2 ml cold 5% trichloroacetic acid (TCA), mixed by tapping, and centrifuged at 300*g* for 10 min. Ascorbic acid standard (0.3 ml) was also added to 5% TCA. Then 0.9 ml of the supernatants or ascorbic acid standard was added to 0.3 ml of dinitrophenylhydrozine–thiourea–copper sulfate reagent (2 g:0.25 g:0.03 g in 100 ml concentrated H_2SO_4), incubated at 37°C for 4 hr, and chilled. Cold 65% H_2SO_4 (1.5 ml) was added to each sample. Samples were incu-

[2] Abbreviations used: PAGE—polyacrylamide gel electrophoresis; Ch—cholesterol; FFA—free fatty acid; ChE—cholesterol ester.

bated at room temperature for 30 min, read on a Beckman spectrophotometer (A_{520}), and compared to the standard.

Histologic preparations. To obtain lung specimens for histologic analysis guinea pigs were killed with an intraperitoneal injection of 150 mg of sodium pentobarbital solution. The chest was opened from the xyphoid process anteriorly up to the mandible along the midline. A small incision was made in the trachea at the larynx and a glass catheter was inserted and tied with surgical thread. The lungs were filled with fixing solution (0.103 M glutaraldehyde, 0.1125 M sodium cacodylate) at a hydrostatic pressure of 20 cm. Lungs were allowed to fix for 30 min and were then removed and trimmed of any extraneous tissues. Tissues were processed by standard paraffin technique for histopathologic evaluation.

Statistical methodology. The data generated from these experiments were analyzed using analysis of variance (ANOVA) and multiple comparison techniques with variation between replicate experiments being accounted for via a blocking factor. Factors of interest in the ANOVA were usually vitamin C deficiency and pollutant exposure. If the univariate F test for one of these factors or if the test for the interaction of the two factors was statistically significant, appropriate multiple comparison tests such as Dunnett's test (Dunnett, 1955) or Duncan's multiple range test (Duncan, 1955) were used to determine the pattern of significant effects. For the protein in lavage fluid data from 5640 and 9400 μg NO_2/m^3 (3 and 5 ppm), all statistical analyses were performed after a logarithmic transformation since Bartlett's (1937) test for homogeneity of variance was rejected.

RESULTS

Characteristics of Vitamin C-Deficient Animals

Table 1 shows blood vitamin C levels obtained from representative experiments. Levels of vitamin C in animals made deficient by feeding on rabbit chow ranged from 14 to 50% of normal and averaged about 25% of normal for experiments reported in this paper. In spite of variability in vitamin C levels obtained

TABLE 1
LEVELS OF VITAMIN C IN BLOOD OF GUINEA PIGS ON NORMAL AND VITAMIN C-DEFICIENT DIETS

Time on diet (weeks)	Air			NO_2		
	Normal diet	C-def. diet	% Normal	Normal diet	C-def. diet	% Normal
2	1.49 ± 0.23[a] (4)[b]	0.21 ± 0.04 (4)	14	1.30 ± 0.15 (4)	0.36 ± 0.03 (4)	27
3	1.45 ± 0.07 (3)	0.37 ± 0.04 (4)	25	0.98 ± 0.09 (4)	0.40 ± 0.02 (2)	41
4	0.73 ± 0.05 (3)	0.18 ± 0.02 (2)	25	0.76 ± 0.07 (3)	0.13 ± 0.03 (3)	17
5	1.55 ± 0.04 (6)	0.28 ± 0.05 (6)	18	1.34 ± 0.22 (5)	0.21 ± 0.04 (5)	16

[a] average mg ascorbic acid/100 ml ± SE.
[b] No. of animals.

from one experiment to the next, the percentage decrease in blood vitamin C of deficient animals compared to controls was constant regardless of whether the animals were held on the diet for 2, 3, 4, or 5 weeks prior to exposure as long as the animal feed came from the same lot. However, substantial differences were noted in the blood vitamin C levels of animals given rabbit feed from different milling dates and some fluctuation was also observed when different lots of guinea pig chow were used. For these experiments efforts were made to standardize by milling date the chow used. Each group of animals was sampled for blood vitamin C levels to ensure that those held on rabbit chow were indeed deficient. Vitamin C-deficient animals used in these experiments did not show obvious weight loss, nor did they exhibit ruffled fur or any other outward signs of impaired health. Exposure of animals to NO_2 did not seem to affect blood vitamin C levels.

72-hr Exposures

Table 2 shows that the protein content of lavage fluids from normal animals exposed to concentrations of NO_2 ranging from 752 to 9400 $\mu g/m^3$ (0.4 to 5 ppm) did not differ from that of air controls. In contrast, the protein content of lavages from vitamin C-deficient animals showed a marked increase over air controls at all but the 752 μg NO_2/m^3 (0.4 ppm) level. The difference over air controls increased

TABLE 2

EXPOSURE TO VARIOUS CONCENTRATIONS OF NO_2 FOR 72 hr: EFFECTS ON PROTEIN CONTENT IN LUNG LAVAGE FLUID FROM VITAMIN C-DEFICIENT AND NORMAL ANIMALS

A. ANOVA

	0.4 ppm		1.0 ppm		3.0 ppm[a]		5.0 ppm[a]	
	F	Prob.	*F*	Prob.	*F*	Prob.	*F*	Prob.
Gas	0.20	0.66	4.78	0.051	31.72	0.0001	105.45	0.0001
Vit. C	1.05	0.31	8.13	0.016	87.10	0.0001	132.58	0.0001
Gas × Vit. C	0.01	0.94	9.99	0.009	27.46	0.0001	110.32	0.0001

B. Means and Standard Errors

		μg Protein/ml			
Gas	Deficient	0.4 ppm	1.0 ppm	3.0 ppm	5.0 ppm
Air	No	336 ± 35 (11)[b]	280 ± 42 (3)	280 ± 17 (4)	375 ± 31 (9)
NO_2	No	318 ± 20 (11)	238 ± 16 (4)	294 ± 21 (4)	374 ± 22 (9)
Air	Yes	372 ± 48 (10)	266 ± 30 (4)	458 ± 34 (5)	429 ± 26 (9)
NO_2	Yes	358 ± 35 (12)	497 ± 65[c] (4)	1977 ± 358[c] (5)	2141 ± 364[c] (4)[d]

[a] All statistical analyses were performed after a logarithmic transformation since the raw data treatment means did not have homogeneous variances.

[b] No. of animals.

[c] This response is significantly different ($P < 0.05$ using Dunnett's test) from that of vitamin C-deficient air-exposed and from that of normal animals exposed to NO_2.

[d] Four other animals died—one sacrificed for pathology.

with dose of NO_2 from twofold at 1880 $\mu g/m^3$ (1 ppm) to fivefold at 9400 $\mu g/m^3$ (5 ppm). In addition, for animals exposed to NO_2, the protein content of lavages from vitamin C-deficient animals was significantly greater ($P < 0.05$) than that of nondeficient animals, except at the lowest NO_2 level (752 $\mu g/m^3$). Statistical analyses of the interaction of vitamin C deficiency and NO_2 exposure showed that the effects of these two on protein content of lavage fluid were synergistic rather than merely additive. During the exposure to 9400 $\mu g/m^3$ (5 ppm), 50% mortality occurred among vitamin C-deficient guinea pigs. Mortalities did not occur in any other test group.

To further analyze the protein content of lavage fluids, PAGE was performed on selected samples of lavage fluids. Figure 1 shows the electrophoresis of lung lavage fluids obtained from normal and vitamin C-deficient animals exposed to air or 5640 μg NO_2/m^3 (3.0 ppm) for 72 hr. Three major protein bands were distinguishable in the lavage fluid from nomral air-exposed animals (A). The electrophoretic profiles of lung lavage fluids from normal animals exposed to 5640 μg NO_2/m^3 (3.0 ppm) for 72 hr (B) or those from vitamin C-deficient animals exposed to air (C) are essentially the same as those of the normal animals. However, lavage fluid from animals that were on a vitamin C-deficient diet for 2 weeks and then exposed to 5640 μg NO_2/m^3 (3.0 ppm) for 72 hr has a very different profile (D). Figure 2 shows spectrophotometric scans of gels A and D. Both the gels and scans

FIG. 1. Polyacrylamide gel electrophoresis of lung lavage fluids from (A) normal animal, (B) vitamin C-deficient animal, (C) normal animal exposed to 5640 μg NO_2/m^3 (3.0 ppm) for 72 hr, (D) vitamin C-deficient animal exposed to 5640 μg NO_2/m^3 (3.0 ppm) for 72 hr, and (E) serum from a normal animal. Extra protein bands present in (D) correspond to some of the protein bands of guinea pig serum (E).

show that there is not only a quantitative increase in the major protein bands, but also a number of new bands. More interestingly, although the third major protein appeared as a single band in all of the gels when they were photographed, spectrophotometric scanning resolved it into three closely migrating but distinct bands (Fig. 2). The quantitative ratio of these three closely migrating bands remains essentially constant in the first three gels (Figs. 1A, B, and C), but in gel D, the ratio is drastically altered and the quantity of the middle band is greatly increased.

The lipid data, shown in Table 3, for 72 hr exposure at selected concentrations of NO_2 are in agreement with the observations for protein. There was no effect in normal animals from NO_2 exposure at 9400 $\mu g/m^3$ (5 ppm); however, significant increases over controls in all assayed lipids were observed in vitamin C-deficient animals at both 5640 and 9400 μg NO_2/m^3 (3 and 5 ppm). The increases for all lipids were on the order of threefold at 9400 μg NO_2/m^3 (5 ppm) and somewhat less than threefold for 5640 μg NO_2/m^3 (3 ppm). Lavage fluids were also analyzed for triglycerides. However, these were present in such small amounts (even in the NO_2, vitamin C-deficient samples) that quantitative analysis was not possible. If extra lipids were the result of serum leaking into the air spaces, one would expect the ratios of Ch/ChE, Ch/FFA, and ChE/FFA in the lavage fluid to approach those in the serum. Therefore, the lavage fluid ratios are included in Table 3. These same ratios were also computed for guinea pig serum, where Ch/ChE = 0.2, Ch/FFA = 0.5, and ChE/FFA = 2.5. The ratio of triglycerides to cholesterol in serum was 10:1. This ratio could not be computed for lavage fluid because so little triglyceride was present.

In an effort to further elucidate the mechanisms responsible for the differences seen in lavage fluid from normal and vitamin C-deficient animals exposed to air and NO_2, lungs from one animal from each of the four groups exposed to 9400 μg

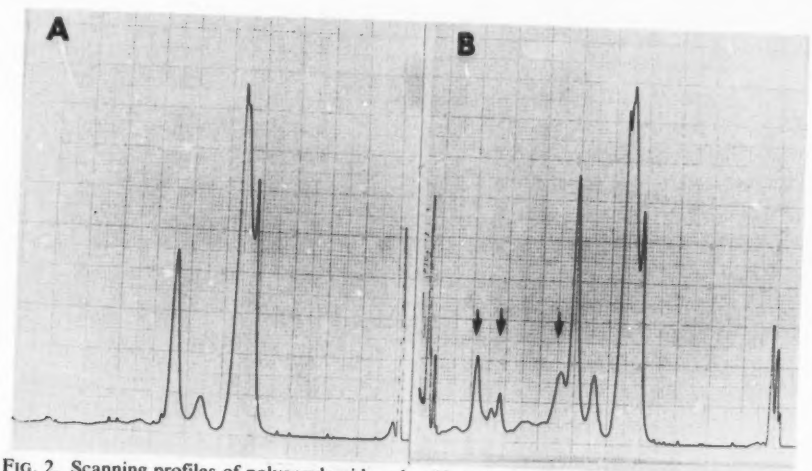

FIG. 2. Scanning profiles of polyacrylamide gels of lung lavage fluids from (A) normal animal and (B) vitamin C-deficient animal exposed to 5640 μg NO_2/m^3 (3.0 ppm) for 72 hr. Note appearance of new protein bands (marked by arrow) in (B).

TABLE 3

EXPOSURE TO VARIOUS CONCENTRATIONS OF NO_2 FOR 72 hr: EFFECTS ON NEUTRAL LIPID PROFILE OF LUNG LAVAGE FLUID FROM NORMAL AND VITAMIN C-DEFICIENT ANIMALS

Gas	Deficient	No. animals	Cholesterol (μg/ml)[a]	Ch/ChE[b]	Cholesterol esters (μg/ml)[a]	Ch/FFA[b]	Free fatty acids (μg/ml)[a]	ChE/FFA[b]
Air	No	9	3.42 ± 0.21	3.81	1.418 ± 0.305	0.822	4.65 ± 0.53	0.301
5 ppm NO_2	No	9	3.11 ± 0.25	4.58	0.839 ± 0.123	0.738	4.39 ± 0.45	0.211
Air	Yes	9	5.05 ± 0.60	6.98	0.713 ± 0.263	0.779	6.85 ± 0.99	0.143
5 ppm NO_2	Yes	4	17.08 ± 3.72[c]	7.42	2.241 ± 0.873[c]	0.804	22.31 ± 5.55[c]	0.129
Air	Yes	5	5.39 ± 0.65	8.55	0.664 ± 0.072	0.951	6.33 ± 1.55	0.128
3 ppm NO_2	Yes	5	14.80 ± 1.94[c]	10.16	1.513 ± 0.154[c]	0.886	17.09 ± 2.37[c]	0.099

[a] Mean ± SE; for 5 ppm NO_2, the ANOVA showed significant ($P < 0.05$) effects of NO_2, vitamin C deficiency, and the interaction between these factors.

[b] Ch = Cholesterol; ChE = cholesterol ester; FFA = free fatty acids.

[c] This response is significantly different ($P < 0.05$ using Dunett's test) from that of vitamin C-deficient, air-exposed, and that of normal animals exposed to NO_2.

NO_2/m^3 (5 ppm) or air were examined for histopathology (Fig. 3). No significant microscopic lesions were present in the lungs from normal animals exposed to either air or NO_2 or in the lungs from the vitamin C-deficient animal exposed to air. However, a multifocal interstitial pneumonia was observed in the lungs from the vitamin C-deficient animal exposed to NO_2. Thickening of alveolar septa was more prominent around terminal bronchioles and alveolar ducts. Numerous macrophages (some of which contained phagocytized debris), a few neutrophils, and proteinaceous edema fluid were in the alveoli. The number of cilia on the epithelial cells lining the terminal bronchioles appeared to be decreased when compared to controls. There was no evidence for bacterial-induced lesions in any of the animals studied regardless of vitamin status or exposure regimen. The histopathologic picture seen in the vitamin C-deficient, NO_2-exposed animal is similar to NO_2-induced pneumonias described by others (Hine *et al.*, 1970; Carson *et al.*, 1962; WHO, 1977) and correlates with the appearance of extra protein in lung lavage fluid from this group of animals. Although a small effect due to vitamin C deficiency alone was detected in lavage fluid protein content, none was detected by histopathology, apparently because the latter method is not sensitive enough to pick up such subtle changes.

1-Week Exposures

In the second exposure regimen, the time of exposure to 752 μg NO_2/m^3 (0.4 ppm) was extended to 7 days. These experiments were done because Sherwin and Carlson (1973) had reported increased protein in lavage fluids following exposure to 752 μg NO_2/m^3 (0.4 ppm) for 1 week, and we were unable to show differences when we exposed animals to this same level of NO_2 for 72 hr. However, even when we extended the 752 μg NO_2/m^3 (0.4 ppm) exposure period to 1 week, we saw no increase in protein concentration (Table 4) or protein species (Fig. 4) in lavage fluids from either normal or deficient animals.

Again, in this experimental regimen as in the last lipid data, Table 5 is for the most part consistent with protein findings. The only statistically significant difference ($P < 0.05$) was in the lower level of free fatty acids in normal NO_2-exposed animals compared to normal controls. The deficient animals at 752 μg NO_2/m^3 (0.4 ppm) appeared to show an increase, although not significant, in lipid levels over deficient controls.

3-hr Exposures

Finally in the third exposure regimen an attempt was made to determine whether deficient animals also exhibited increased protein levels when the time of exposure to 9400 μg NO_2/m^3 (5 ppm) was reduced to 3 hr. Figure 5 shows that animals exposed in this manner and lavaged immediately after exposure did not show significant increases in lavage protein concentration. However, if the time of lavage was delayed until 15 hr after the exposure, significant differences between NO_2- and air-exposed animals were observed. While not significantly different from air controls, protein concentrations in lavages obtained 12, 16, 19, and 24 hr after NO_2 exposure were significantly different from those taken 5 and 10 hr after exposure (Duncan's test).

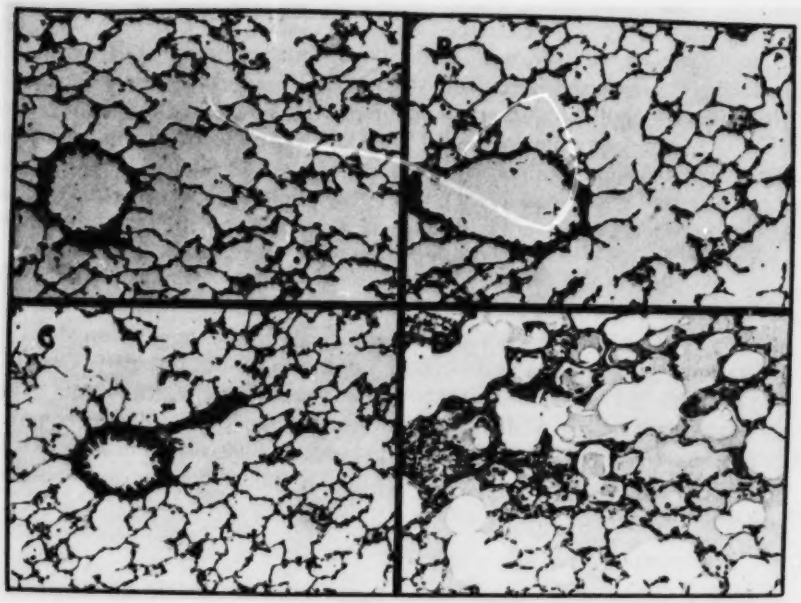

FIG. 3. Lungs of normal and vitamin C-deficient guinea pigs exposed to 9400 μg NO_2/m^3 (5.0 ppm) or filtered air for 72 hr. (A) Normal and filtered; (B) vitamin C-deficient and filtered air; (C) normal and NO_2 exposed; (D) vitamin C-deficient and NO_2 exposed. Note the thickening of alveolar wall due to interstitial pneumonia and accumulation of proteinaceous edema fluid in alveoli in the lung of vitamin C-deficient animal following NO_2 exposure (300×)

DISCUSSION

The present study investigated protein and lipid accumulation in lavage fluid of guinea pigs as indicators of toxicity following inhalation exposure to NO_2. Large differences were seen between vitamin C-deficient and normal guinea pigs exposed to NO_2. When compared to air-exposed controls, no increases in protein concentrations were observed in any of the normal animals exposed for 72 hr to concentrations of NO_2 ranging from 752 to 9400 $\mu g/m^3$ (0.4 to 5.0 ppm). Similarly, no increases were observed in free fatty acid (FFA), cholesterol (Ch), or cholesterol ester (ChE) concentrations. In contrast, vitamin C-deficient animals exposed

TABLE 4
EFFECTS OF 1 week EXPOSURE TO 0.4 ppm NO_2 ON PROTEIN CONTENT OF LUNG LAVAGE FLUID FROM VITAMIN C-DEFICIENT AND NORMAL ANIMALS

Gas	Deficient	No. animals	μg protein/ml[a]
Air	No	16	290 ± 22
NO_2	No	15	326 ± 21
Air	Yes	15	318 ± 25
NO_2	Yes	16	314 ± 15

[a] The data are means ± SE. No effects were found to be significant in the two-way ANOVA.

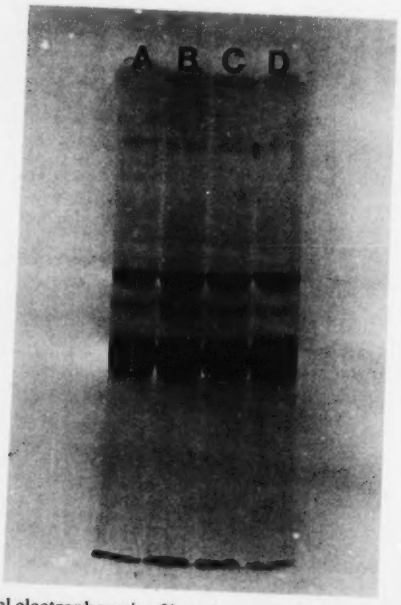

FIG. 4. Polyacrylamide gel electrophoresis of lung lavage fluids from (A) normal animal, (B) vitamin C-deficient animal, (C) normal animal exposed to 752 μg NO_2/m^3 (0.4 ppm) for 1 week, and (D) vitamin C-deficient animal exposed to 752 μg NO_2/m^3 (0.4 ppm) for 1 week. There is no difference in the electrophoretic profiles of all groups.

to 1880, 5640, or 9400 μg NO_2/m^3 (1, 3, or 5 ppm) for 72 hr showed two- to fivefold increases in the concentrations of proteins and lipids as well as new protein species on polyacrylamide gels. At 9400 μg NO_2/m^3 (5 ppm) 50% of vitamin C-deficient animals died and histologic examination of the lungs of a surviving animal revealed severe edema. This was in sharp contrast to air controls and normal animals exposed to NO_2 since no ill effects could be demonstrated in these groups.

This study also demonstrates that analysis of lipid and protein content in lung lavage fluid provides a sensitive method for determining subtle changes in the lung following exposure to NO_2. At 9400 μg NO_2/m^3 (5 ppm) for 72 hr these changes correlated with more obvious health effects—mortality and alveolar edema. When vitamin C-deficient animals were exposed to 9400 μg NO_2/m^3 (5 ppm) for 3 hr, significant differences from air controls were seen only when the lavage time was delayed until 15 hr after exposure, indicating that some time is required for the damage to be expressed in terms of increased protein in lung lavage fluid. This type of delayed effect has also been observed with the appearance of edema following O_3 exposure (Scheel *et al.*, 1959).

When vitamin C-deficient as well as normal animals were exposed to 752 μg NO_2/m^3 (0.4 ppm), no differences in protein, Ch, or ChE were seen when compared to air controls, even when the exposure period was extended to 1 week. This is in contrast to the work of Sherwin and Carlson (1973), who reported

TABLE 5

EFFECTS OF 1 week EXPOSURE TO 0.4 ppm NO_2 ON NEUTRAL LIPID PROFILE OF LUNG LAVAGE FLUID FROM NORMAL AND VITAMIN C-DEFICIENT ANIMALS

A. ANOVA

	Cholesterol (Ch)		Cholesterol esters (ChE)		Free fatty acids (FFA)		Ch/ChE		Ch/FFA		ChE/FFA	
	F	Prob.	*F*	Prob.	*F*	Prob.	*F*	Prob.	*F*	Prob.	*F*	Prob.
Gas	0.04	N.S.[a]	0.45	N.S.	4.57	0.054	0.25	N.S.	1.42	N.S.	1.19	N.S.
Vit. C	0.70	N.S.	1.04	N.S.	12.19	0.0005	2.76	N.S.	2.51	N.S.	23.20	0.0005
Gas × Vit. C	1.85	N.S.	2.13	N.S.	1.92	N.S.	0.03	N.S.	0.61	N.S.	2.38	N.S.

B. Means and Standard Errors

Gas	Deficient	Cholesterol (μg/ml)	Cholesterol esters (μg/ml)	Free fatty acids (μg/ml)	Ch/ChE	Ch/FFA	ChE/FFA
Air	No	3.84 ± 0.68[b]	3.21 ± 1.14	12.05 ± 2.00	1.77 ± 0.57	0.340 ± 0.06	0.246 ± 0.06
NO_2	No	3.07 ± 0.12	1.66 ± 0.26	7.78 ± 0.27	2.12 ± 0.58	0.396 ± 0.02	0.214 ± 0.03
Air	Yes	2.75 ± 0.13	2.89 ± 0.49	6.18 ± 0.47	1.01 ± 0.17	0.450 ± 0.04	0.482 ± 0.11
NO_2	Yes	3.33 ± 0.58	3.46 ± 0.53	5.25 ± 0.84	1.17 ± 0.47	0.724 ± 0.24	0.671 ± 0.08

[a] N.S. = Not significant, $P > 0.1$.

[b] Data given are mean ± SE; there were four animals/group except for the vitamin C-deficient, air-exposed group where there were three animals.

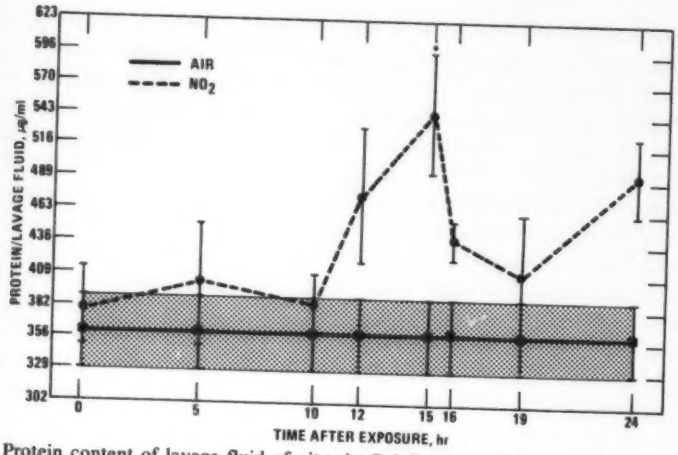

FIG. 5. Protein content of lavage fluid of vitamin C-deficient animals exposed to air or 9400 μg NO_2/m^3 (5.0 ppm) for 3 hr and sacrificed at various intervals after exposure. The (*) indicates a significant difference from control as determined by Dunnett's test. No significant difference was found between the protein concentration of fluids obtained at 12, 15, 16, 19, and 24 hr using Duncan's test, but these were significantly higher than protein contents obtained at 5 and 10 hr.

increases in lavage fluid protein content in vitamin C-deficient animals exposed for 1 week at this level. Methods for analyzing and thus controlling NO_2 concentrations have improved since Sherwin and Carlson's study and could explain the differences between their study and ours. Also the level of vitamin C deficiency was not monitored in their study and could have been quite different from that in our study. Finally, they used PAGE to quantitate their protein concentrations whereas we used PAGE only for qualitative purposes and used the Lowry method for quantitation. A protein standard was employed in all our Lowry assays so that comparisons could be made between assays made on different days.

Additional experiments are needed to identify the mechanisms responsible for the increases in protein and lipid in lung lavage fluid seen in the present study. Increased lung permeability due to NO_2 exposure with subsequent leakage of serum proteins is one possibility. Long-term chronic exposure to low doses of NO_2 and acute exposure to high doses of NO_2 have been shown to produce edema (Hine *et al.*, 1970; Nakajima *et al.*, 1972; Klinerman and Rynbrandt, 1976). Sherwin and Richters (1971), after injecting mice with tritiated serum proteins, reported higher levels of radioactivity in lungs of mice exposed to 7520–13,160 μg NO_2/m^3 (4–7 ppm) for 72 hr than in those of air controls. Alpert *et al.* (1971) reported similar results when rats were injected with ^{131}I-albumin and exposed to O_3, a photo oxidant similar to NO_2. All these observations suggest that NO_2 can cause damage to the respiratory epithelium which could result in increased permeability. Alternately, increased proteins and lipids could be the result of hypersecretion by various cells lining the lung or excessive damage to these cells. Vitamin C has been shown to protect against toxicity of O_3. Both edema and death were reduced when animals were treated with ascorbic acid. It was suggested that the effect of ascorbic acid

was due either to the prevention of release of histamine which would subsequently cause cell damage or to neutralization of free radicals and peroxides also responsible for lung damage (Mittler, 1958; Ungar and Bolgert, 1938; Matzen, 1957).

In the present study the appearance of new species of proteins on PAGE after exposure to the higher concentrations of NO_2 and the pathological effects observed at 5 ppm suggest serum leakage. However, if serum is the source of additional protein, the lipid data argue for a molecular sieving mechanism which is highly discriminating in allowing only small protein or polypeptide species to pass through. If leakage of serum components were occurring into the lung through effective pore sizes large enough to allow all lipoprotein classes access, then one would expect a dramatic increase in the triglyceride levels as well as decreases in the ratios of Ch/ChE, Ch/FFA, and ChE/FFA. This was not observed. From our data it appears that NO_2 produces damage in the lungs of vitamin C-deficient animals which results in selective leakage of serum components into the lung.

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